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Xenopus Mad Proteins Transduce Distinct Subsets of Signals for the TGFβ Superfamily

Jonathan M. Graff, Anu Bansal, and Douglas A. Melton
Howard Hughes Medical Institute
Department of Molecular and Cellular Biology
Harvard University
Cambridge, Massachusetts 02138

Summary

Xenopus cDNAs homologous to the Drosophila *Mad* gene and C. elegans *CEM* genes have been cloned and functionally analyzed by microinjection into frog embryos. The results show that these genes (*Xmad*) encode intracellular proteins that act downstream of TGFβ superfamily ligands. Most interesting is the fact that different Xmad proteins produce distinct biological responses. Xmad1 produces ventral mesoderm, apparently transducing a signal for BMP2 and BMP4, whereas Xmad2 induces dorsal mesoderm like Vg1, activin, and nodal. These results suggest that an individual Xmad protein waits poised in the cytoplasm for instruction from a distinct subset of TGFβ ligands and then conveys specific information to the nucleus.

Introduction

The transforming growth factor β (TGFβ) superfamily consists of more than 25 related proteins from insects to man and includes the bone morphogenetic proteins (BMPs), activin, inhibin, Müllerian inhibiting substance, nodal, glial-derived neurotropic factor, Decapentaplegic (DPP), and Vg1 as well as the TGFβs. These signals mediate a very diverse array of biological processes, including immune function, growth control, cell differentiation, sexual reproduction, skeletal formation, and patterning the embryonic body (reviewed by Kingsley, 1994; Wall and Hogan, 1994; Massagué et al., 1994). Yet, unlike what is known for many other cytokines, such as fibroblast growth factors, Wnts, insulin, and adrenaline, the cytoplasmic components of the TGFβ signal transduction cascade are essentially unknown (reviewed by Derynck, 1994; Kingsley, 1994; Wall and Hogan, 1994; Massagué et al., 1994).

Initial insights into how TGFβ family members transduce their signal were obtained by cloning their receptors. The receptor complex consists of at least two distinct transmembrane serine/threonine kinases, indicating that protein phosphorylation is involved (Massagué et al., 1994; Wrana et al., 1994; Mathews, 1994). Efforts aimed at elucidating the downstream cytoplasmic elements of the signaling cascade have not been as successful (see Wall and Hogan, 1994; Derynck, 1994; Mathews, 1994). A few candidates have been obtained by biochemical means and yeast two-hybrid cloning, although there is no evidence yet to establish them as functioning in TGFβ signal transduction (Wang et al., 1994; Chen et al., 1995; Yamaguchi et al., 1995).

The best candidate to date for an intracellular component of the TGFβ signal transduction pathway is the Drosophila gene *Mothers against dpp*, or *Mad* (Raftery et al., 1995; Sekelsky et al., 1995). Gelbart and colleagues identified *Mad* by a suppressor/enhancer screen for genes interacting with DPP during fly embryogenesis. Recently, it has been shown that the Drosophila MAD protein is present in the cytoplasm and is required for any response to DPP by visceral mesoderm or endodermal cells (Newfeld et al., 1996). Moreover, we found that the Drosophila MAD protein functions in a TGFβ-responsive assay for mesoderm induction in Xenopus, implying that a *Mad*-related gene is present in vertebrates and would be part of the TGFβ signaling cascade (Newfeld et al., 1996). Conservation of a family of *Mad*-related genes in flies and worms has also been demonstrated (Savage et al., 1996).

Here we describe the cloning of two functionally distinct Xenopus *Mad*-related genes. The most significant finding reported here is that different Xenopus *Mad* proteins (Xmads) generate strikingly distinct biological responses. In all, our data suggest that Xmads are intracellular components of TGFβ superfamily signaling and that individual Xmad proteins are dedicated to transducing the signals for a specific subclass of TGFβ ligands. As such, the Xmads provide the ability to activate specific TGFβ pathways cell autonomously, a novel reagent to help understand how TGFβ family signaling works, and an intracellular target for therapeutics.

Results

The *Xmads* Are a Family of Genes

Degenerate polymerase chain reaction (PCR) primers were used to screen a Xenopus oocyte library, and four different *Xmad* cDNAs were cloned, two of which are characterized here. The sequences of Xmad1 and Xmad2 are shown in Figure 1. Xmad1 is 76% identical to MAD and 62% identical to Xmad2. This high degree of sequence conservation suggests that the *Xmads* are vertebrate homologs of the Drosophila *Mad* gene. In addition, the vertebrate Xmads are homologous to three MAD-related Caenorhabditis elegans sequences, called C. elegans MAD (CEM)-1, CEM-2, and CEM-3, identified in the C. elegans genome sequencing project (Sekelsky et al., 1995; Savage et al., 1996). Xmad2 contains an alternatively spliced exon, which appears to be present at the identical position in CEM-3 (Sekelsky et al., 1995). In cloning of frog, mouse, and human cDNAs or genes, six different *Xmads* have been identified to date, and they appear to fall into four classes that correspond closely to the sequences identified in invertebrates (J. M. G. and D. A. M., unpublished data). The open reading frames predict proteins with molecular weights between 50,000 and 55,000 Da that contain no signal sequence, transmembrane domain, or obvious homology to other known protein sequence motifs.

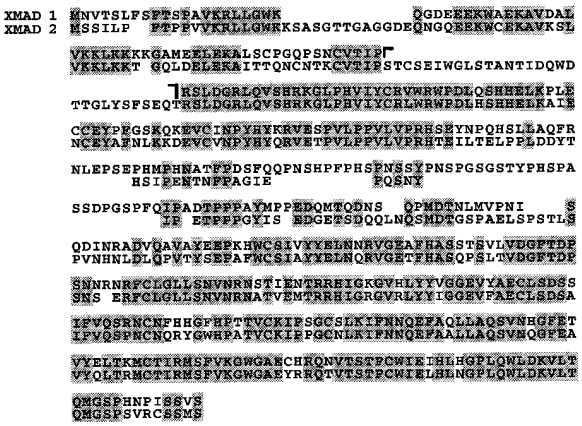

Figure 1. Amino Acid Sequences of Xmad1 and Xmad2

Alignment of the predicted protein sequences of Xenopus Xmad1 and Xmad2. Identical residues are indicated by a stippled background. Two marks enclose an alternatively spliced exon in Xmad2.

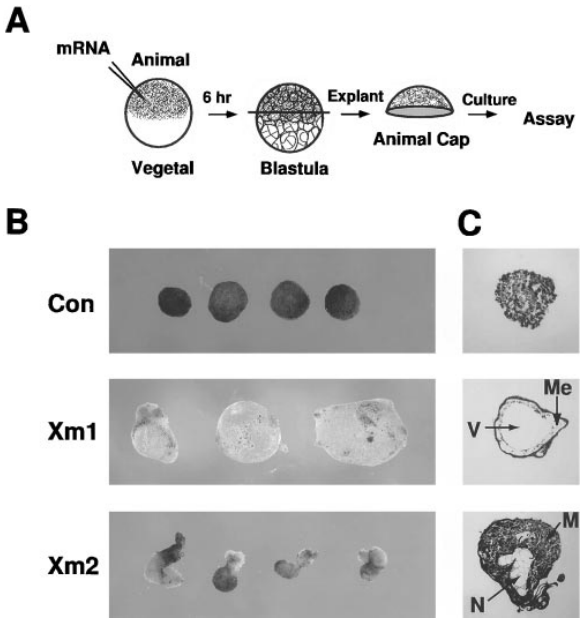

Figure 2. An Individual Xmad Transmits a Distinct Subset of TGFβ Signals

(A) Experimental design to assay Xmad function in animal caps. Synthetic mRNAs encoding a Xmad protein were injected into animal poles of fertilized eggs. At the blastula stage, animal caps were dissected and cultured in salt buffer.

(B) Morphological assay for Xmad function. Control (Con) animal caps are round balls of ciliated epidermis, photographed at the equivalent of stage 18. Injection of 2 ng of *Xmad1* RNA (Xm1) directs formation of ventral mesoderm, as demonstrated by the large fluid-filled vesicles, photographed at the equivalent of tadpole stage 38 when these vesicles are most pronounced. *Xmad2*-expressing caps (Xm2; 2 ng of RNA per egg) undergo morphogenetic movements and elongate, characteristic of dorsal mesoderm; they are photographed at the equivalent of stage 18.

(C) Histological sections of the animal caps shown in (B). Control caps develop into atypical epidermis, an ectodermal derivative. Xmad1-expressing caps have vesicles (V), mesenchyme, and mesothelium (Me). Elongated *Xmad2*-injected caps contain muscle (M) and notochord (N).

Xmads Function in Discrete Pathways

Xenopus laevis animal pole explants normally become ectoderm (ciliated epidermis), but can be converted into either dorsal or ventral mesoderm depending on which TGFβ superfamily ligand is used as an inducer. Activin, Vg1, TGFβ, and nodal all induce dorsal mesoderm (Rosa et al., 1988; Thomsen et al., 1990; Green et al., 1990; Dale et al., 1993; Thomsen and Melton, 1993; Jones et al., 1995), whereas BMP2 and BMP4 induce ventral mesoderm (Koster et al., 1991; Dale et al., 1992; Jones et al., 1992; Hemmati-Brivanlou and Thomsen, 1995). These two types of mesoderm, dorsal or ventral, are easily distinguished by morphology, histology, and molecular markers. To test whether direct expression of the Xmads induces mesoderm (sends a TGFβ-like signal), synthetic mRNAs encoding a Xmad protein were injected into the animal poles of fertilized eggs, and animal caps were removed, cultured, and then assayed for mesoderm induction (Figure 2A). When Xmad1 is expressed in an animal pole explant, ventral mesoderm forms, as evidenced by fluid-filled vesicles (Figure 2B) containing mesenchyme and mesothelium (Figure 2C). Animal caps injected with *Xmad1* do not express the dorsal mesodermal markers goosecoid or muscle actin or the neural marker, neural cell adhesion molecule (N-CAM), but do express globin, a definitive marker of ventral mesoderm (Figure 3). Unexpectedly, formation of ventral mesoderm by Xmad1 occurs in the absence of expression of the early markers for mesoderm, such as brachyury (Figure 3). In all, these data show that Xmad1 induces the same type of mesoderm (ventral) that is observed when animal caps are induced by BMP2 or BMP4 (Koster et al., 1991; Dale et al., 1992; Jones et al., 1992; Hemmati-Brivanlou and Thomsen, 1995).

In contrast, when Xmad2 is expressed in the animal pole, the tissue elongates in a manner characteristic of dorsal mesoderm (Figure 2B), and histological analyses demonstrate the presence of muscle and notochord (Figure 2C). This is confirmed by immunohistochemistry with a muscle-specific monoclonal antibody, 12/101, and a notochord-specific antibody, Tor70.1 (data not shown). Molecular analysis demonstrates that mesoderm induced by Xmad2 does not express the ventral marker globin, but does express the dorsal markers goosecoid and muscle actin (Figure 3). Therefore, Xmad2, like activin, Vg1, TGFβ, and nodal, induces dorsal mesoderm. Thus, Xmad1 and Xmad2 produce two distinct and easily distinguished biological responses: Xmad1 produces ventral mesoderm and Xmad2 produces dorsal mesoderm.

To demonstrate further that the distinct responses seen with Xmad1 and Xmad2 are qualitative differences and not concentration-dependent differences, we assayed the two Xmads at doses ranging from 15.6 pg to 2 ng (Figure 4). Xmad2 induces mesoderm over a broad range of doses from 125 pg to 2 ng (Figure 4A) and can induce mesoderm formation at a dose of 60 pg (data not shown). Higher concentrations of Xmad2 induce expression of goosecoid, a marker for the most dorsal mesoderm. At lower Xmad2 concentrations, goosecoid is not expressed, but the ventral–lateral marker Xwnt-8 is expressed. Significantly, no concentration of Xmad2

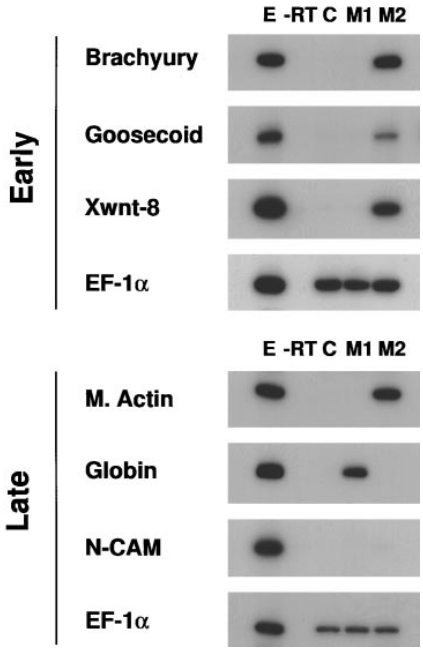

Figure 3. Induction of Mesodermal Gene Markers as an Assay of Xmad Function

Animal caps injected with 2 ng of either *Xmad1* (M1) or *Xmad2* (M2) RNA were cultured until either gastrula stage 10.5 (Early) or tadpole stage 38 (Late), and total RNA was harvested. RNA was analyzed by RT–PCR for the presence of the indicated transcripts. Xmad1 induces the expression of the ventral mesodermal marker globin and none of the dorsal markers. In contrast, Xmad2 induces the expression of the dorsal markers goosecoid and muscle actin (M. Actin) and does not induce the expression of the ventral marker globin. EF-1α, ubiquitously expressed, is a loading control (Krieg et al., 1989). RNA from embryos (E) provides a positive control. The −RT lane is identical to the embryo lane, except reverse transcriptase was omitted and serves as a negative control. Lane C corresponds to control animal caps and demonstrates that mesodermal markers are not normally expressed in the animal cap. Brachyury is a marker of general mesoderm (Smith et al., 1991a). Goosecoid is a marker of dorsal mesoderm (Cho et al., 1991). Xwnt-8 is a marker of ventral and lateral mesoderm (Christian et al., 1991; Smith and Harland, 1991). Muscle actin is a marker for the dorsal and lateral tissue, muscle (Mohun et al., 1984). Globin is a marker of blood and is a definitive ventral marker (Hemmati-Brivanlou et al., 1990). N-CAM is a marker of neural tissue (Kintner and Melton, 1987).

leads to the expression of the ventral marker globin. These results reproduce the concentration effects obtained with varying doses of activin and Vg1, TGFβ molecules that induce dorsal mesoderm (Green et al., 1990, 1992; Wilson and Melton, 1994; Kessler and Melton 1995).

The results obtained with Xmad1 contrast with those produced by Xmad2 (Figure 4B). At no dose does Xmad1 induce any of the dorsal markers goosecoid, actin, or N-CAM, but Xmad1 does induce expression of globin, mimicking BMP2 and BMP4. In addition, Xmad1 appears to be much less potent than Xmad2, requiring nanogram quantities of mRNA to produce mesoderm. This too mimics the effects seen with the ligands, as BMPs are less potent than either activin or Vg1 (Thomsen et al., 1990; Thomsen and Melton, 1993; Hemmati-Brivanlou and Thomsen, 1995).

When mRNAs encoding Xmad1 and Xmad2 are coinjected, both dorsal and ventral mesoderm are induced. Figure 4C shows that coinjection leads to induction of the dorsal marker muscle actin and the ventral marker globin.

Taken together, these data demonstrate that Xmad1 induces ventral mesoderm, mimicking the effects of BMP2 and BMP4, whereas Xmad2 induces dorsal mesoderm, mimicking the effects of dorsal-inducing ligands such as activin and Vg1. Thus, the Xmad proteins have qualitatively distinct activities in embryonic mesoderm induction.

Xmads Are Uniformly Expressed during Embryonic Development

Since individual Xmads induce either ventral or dorsal mesoderm, but not both, their localization or differential activation could explain how embryonic mesoderm is initially established and patterned. We determined the spatial distribution of the *Xmad* transcripts in various regions of developing embryos by reverse transcription–PCR (RT–PCR). *Xmad* RNAs are maternally expressed, since the cDNAs were recovered from an oocyte library. The RNAs are present in the blastula stage, and both *Xmad1* and *Xmad2* mRNAs are present in all blastula regions and at approximately equal levels (Figure 5). Similarly, during early gastrulation, *Xmad1* and *Xmad2* mRNAs appear to be equally distributed in the ventral and dorsal marginal zones (Figure 5). A time course of *Xmad1* and *Xmad2* expression shows that the RNAs are present at a nearly constant level from the 2-cell stage to the tadpole stage (data not shown). The spatial and temporal constancy during the formation of dorsal–ventral mesodermal pattern suggests that distinct TGFβ signals activate different Xmad proteins on different sides of the embryo.

To test whether mesoderm induction by TGFβ superfamily ligands affects transcription of *Xmad* genes, we added BMP4 or activin protein to ectodermal explants and analyzed *Xmad* mRNA levels at 40 min intervals until mesoderm was induced. As expected, both BMP4 and activin induce mesoderm, assayed here by expression of brachyury RNA at 160 min (Figure 5). The level of *Xmad1* and *Xmad2* mRNA is unaffected at all four timepoints (Figure 5), suggesting that transcription of *Xmad1* and *Xmad2* is not significantly altered by mesoderm induction. In all, these data indicate the presence of a nearly uniform and constant amount of *Xmad1* and *Xmad2* mRNAs in early development.

Xmads Function Downstream of the Receptor

We tested the position of the Xmads within the TGFβ signaling cascade using truncated receptors that function as dominant-negative receptors. One expects signals that function upstream of the receptor to be blocked by a truncated receptor, whereas signals acting downstream of the receptor might be unaffected (Herskowitz, 1987; Amaya et al., 1991; Hemmati-Brivanlou and Melton, 1992; Graff et al., 1994; Suzuki et al., 1994; Umbhauer et al., 1995). Xmad1 appears to be located in the BMP-specific pathway, and the truncated BMP receptor does not affect the Xmad1-dependent morphologic or

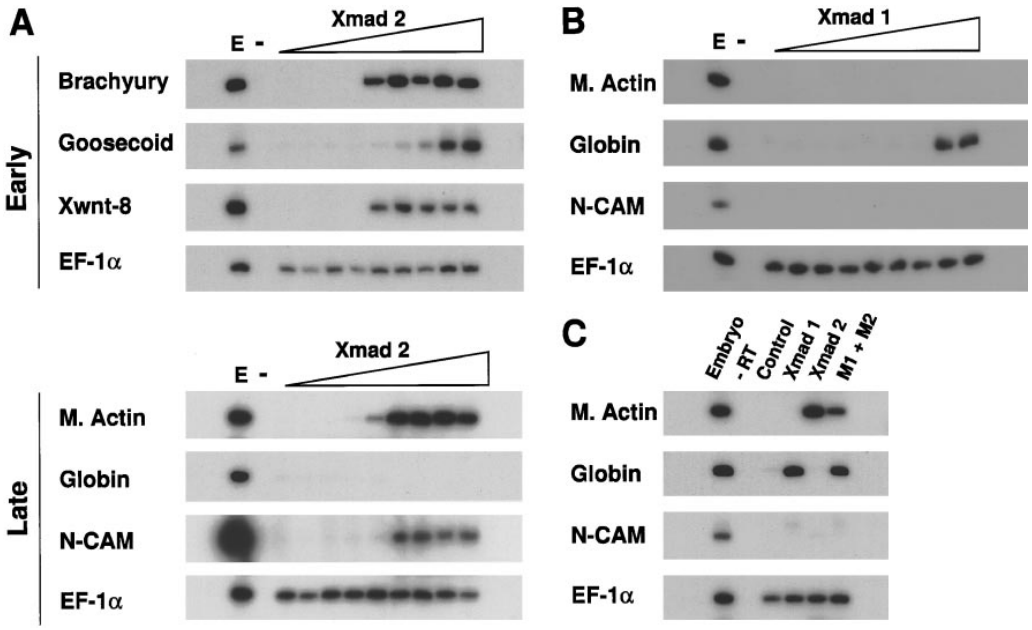

Figure 4. Dose-Dependent Induction of Mesoderm by Xmads

(A) Animal poles expressing different amounts of Xmad2 were cultured until either gastrula stage 11 (Early) or tadpole stage 38 (Late), and total RNA was harvested. RNA was analyzed by RT–PCR for the presence of the indicated transcripts. Xmad2 was expressed in a 2-fold dilution series from 2 ng to 15.6 pg. Xmad2 induces the expression of the different molecular markers beginning at about 125 pg of RNA in a concentration-dependent manner. Significantly, Xmad2 never induces the expression of the ventral marker globin. The markers and lanes are as described in the legend to Figure 3, except that the negative control is labeled with a minus sign.

(B) Xmad1 only induces the expression of ventral mesoderm, not dorsal mesoderm. Animal poles expressing different concentrations of Xmad1 were cultured until the tadpole stage 38, and total RNA was harvested. The concentrations of Xmad1 and the analysis is as described in (A). Note the absence of muscle actin expression (dorsal mesoderm), even at high doses.

(C) Coexpression of Xmad1 and Xmad2 leads to formation of ventral and dorsal mesoderm. Animal caps expressing Xmad1 (2 ng), Xmad2 (2 ng), or Xmad1 and Xmad2 (M1 + M2; 2 ng of each) were cultured until tadpole stage 38, and total RNA was harvested. Xmad1 induces expression of the ventral marker globin, Xmad2 induces the expression of the dorsal marker actin, and the combination leads to expression of both markers. The analysis is as described in (A).

histologic induction of ventral mesoderm, as evidenced by the fact that vesicles, mesenchyme, and mesothelium form unabated when Xmad1 is coexpressed with the dominant-negative BMP receptor (Figure 6A). In contrast with this lack of effect on morphology and histology, the truncated BMP receptor does block the Xmad1-dependent induction of globin (Figure 6B). The formation of vesicles, mesenchyme, and mesothelium is an early and potentially direct effect of expression of Xmad1 (and BMP signaling), whereas expression of globin is a late effect that presumably requires many steps, and the truncated BMP receptor may alter a later step without blocking Xmad1 function per se. The blockade of globin expression might also be explained by the truncated BMP receptor inhibiting endogenous BMP signaling present in animal caps (Graff et al., 1994; Suzuki et al., 1994; Hawley et al., 1995; Sasai et al., 1995; Schmidt et al., 1995; Wilson and Hemmati-Brivanlou, 1995). If ectopic expression of Xmad1 requires endogenous BMP activity to induce globin, then the truncated BMP receptor may eliminate globin expression by blocking endogenous BMP signaling. In support of this interpretation, coexpression of BMP4 and *Xmad1* mRNA in quantities that on their own have no effect leads to induction of globin (data not shown).

Another way to determine if Xmad1 is downstream of receptors is to test whether Xmad1 can reverse phenotypic effects of the truncated dominant-negative receptors. The truncated BMP receptor, which blocks BMP signaling, leads to a weak induction of neural tissue, as demonstrated by the induction of N-CAM (Figure 6C) (Sasai et al., 1995; Hawley et al., 1995). Similarly, the truncated activin receptor, which blocks all tested TGFβ signals including BMPs, induces neural tissue and does so more potently than the truncated BMP receptor (Figure 6C) (Hemmati-Brivanlou and Melton, 1992; Schulte-Merker et al., 1994; Kessler and Melton, 1995; Hemmati-Brivanlou and Thomsen, 1995). Xmad1 completely reverses the induction of N-CAM by either of the truncated receptors, implying that Xmad1 functions downstream of the receptor. This reversal of N-CAM expression is not seen when BMP4 is coexpressed with the truncated BMP receptor (Sasai et al., 1995).

Xmad2 appears to function in the activin/Vg1-like dorsal pathway, so we determined whether the dominant-negative activin receptor would block Xmad2 function. The truncated activin receptor blocks activin and Vg1 function and formation of all dorsal mesoderm (Hemmati-Brivanlou and Melton, 1992; Schulte-Merker et al., 1994; Kessler and Melton, 1995). Microinjection of the

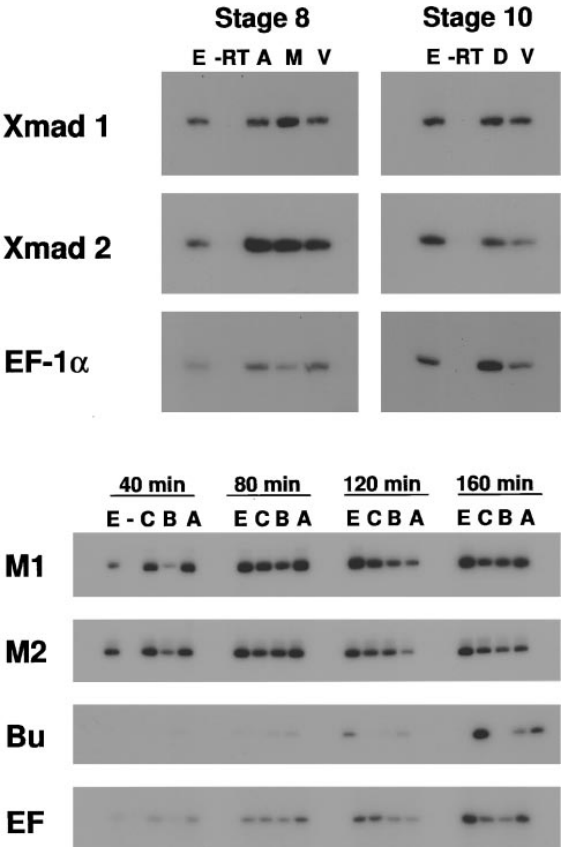

Figure 5. Expression of the *Xmad* RNAs during Xenopus Development

(Top) *Xmad* transcripts are uniformly expressed in early Xenopus embryos. Stage 8 blastula were dissected into roughly equal thirds (animal [A], marginal [M], or vegetal [V]), and total RNA was harvested. At stage 10, dorsal (D) and ventral (V) marginal zones were explanted, and total RNA was harvested. The RNA was analyzed by RT–PCR for the presence of the *Xmad1*, *Xmad2*, and EF-1α transcripts. The other control lanes are as described in the legend to Figure 3.

(Bottom) Expression of Xmads is not affected by mesoderm induction. Blastula-stage animal caps were dissected and cultured in control buffer (C), 130 nM BMP4 protein (B), or 2.3 nM activin protein (A). RNA was harvested at 40 min intervals (the last timepoint is equivalent to early gastrula, stage 10.5) and analyzed by RT–PCR for the presence of the *Xmad1* (M1), *Xmad2* (M2), brachyury (Bu), and EF-1α (EF) transcripts. The other control lanes are as described in the legend to Figure 3, except that the negative control is labeled with a minus sign.

truncated activin receptor leads to expression of N-CAM, which demonstrates that the dominant-negative activin receptor is active (Figure 6D) (Hemmati-Brivanlou and Melton, 1992). Coexpression of the dominant-negative activin receptor with Xmad2 does not block the morphogenetic elongation induced by Xmad2 (data not shown). Furthermore, the dominant-negative activin receptor has no effect on mesoderm formed by Xmad2, as demonstrated by the lack of effect on the molecular markers brachyury and muscle actin (Figure 6D). These results support the contention that Xmads function downstream of the receptors.

Xmads Are Present in the Cytoplasm and the Nucleus

To determine the subcellular location of Xmad proteins, we microinjected oocytes with synthetic mRNA encoding either Xmad1 or Xmad2 and incubated the oocytes with ^{35}S-containing amino acids. Newly synthesized proteins were assayed from oocyte culture media (containing secreted proteins), manually isolated nuclei, and biochemically fractionated membranes and cytoplasm. Gel fractionation of newly synthesized proteins (Figure 7) shows that the Xmad proteins are present in both the nucleus and cytoplasm, but they are not in the membrane fraction or secreted into the media. Close inspection of the nuclear and cytoplasmic lanes reveals that the nuclear Xmad protein appears slightly larger. This reproducible effect suggests that the nuclear protein may be posttranslationally modified. To eliminate the possibility that the nuclear or cytosolic localization of Xmads is due to overexpression, we expressed Xmads at lower concentrations and determined their subcelluar location by Western blotting. When the Xmads were expressed at the detection limit of the antibody (20- to 100-fold less mRNA than that used in Figure 7), the protein is still found in both the cytosol and nucleus.

Discussion

The results presented here show that the Xmads are components of the vertebrate TGFβ signaling pathway. Expression of individual Xmad proteins mimics the effects of specific subsets of TGFβ signals in mesoderm induction in Xenopus by producing dorsal or ventral mesoderm. Moreover, experiments showing that the truncated receptors do not block Xmad signaling combined with epistatic tests demonstrating genetically a requirement for Mad in cells responding to DPP (Newfeld et al., 1996) support the contention that Xmads are downstream of the ligands and receptors in the TGFβ signal transduction cascade.

Consistent with this view are the immunohistochemical studies with the Drosophila MAD protein (Newfeld et al., 1996) and biochemical fractionation in Xenopus oocytes showing that the Xmads are intracellular proteins. The data presented in Figure 6 suggest that there may be a difference between the nuclear and cytoplasmic forms of the Xenopus Xmad proteins. Given the precedent of other signal transduction cascades, it is possible that a ligand-dependent change leads to translocation of Xmad proteins from one compartment to the other (Verma et al., 1995). As the Xmads are part of a signaling cascade initiated by a receptor serine/threonine kinase, it is feasible that the size difference between the nuclear and cytosolic versions is accounted for by phosphorylation. Indeed, preliminary experiments suggest that the Xmads are phosphoproteins (J. M. G., P. J. Blackshear, D. J. Stumpo, and D. A. M., unpublished data).

Xmad1 appears to transduce the BMP set of signals for ventral mesoderm induction, whereas Xmad2 transduces the activin/Vg1/Nodal/TGFβ signals to form dorsal mesoderm. Thus, it appears that the Xmads act as

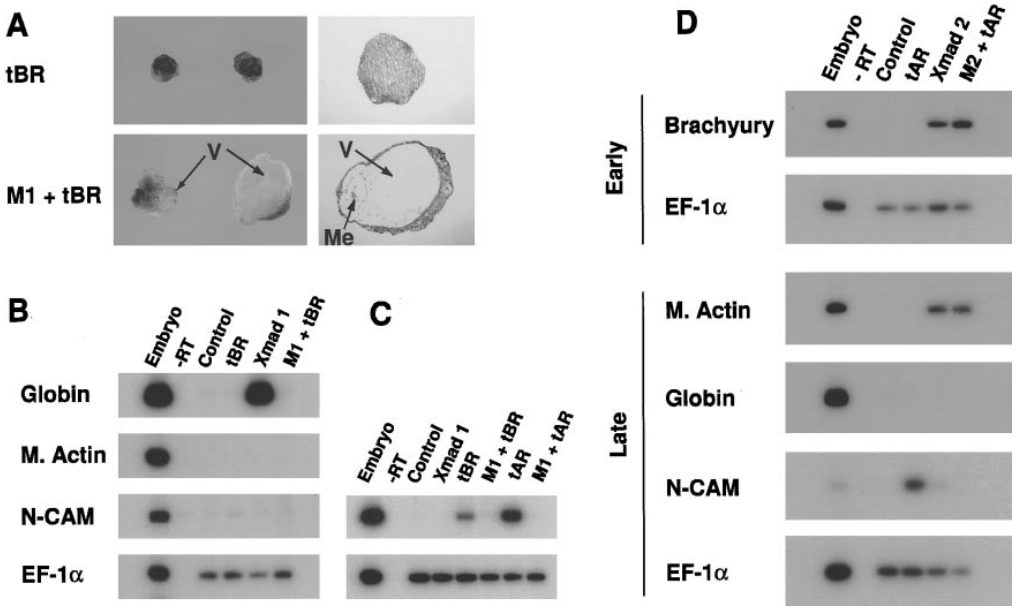

Figure 6. Xmads Function Downstream of the Receptor

(A) The dominant-negative BMP receptor does not block Xmad1 induction of ventral embryonic mesoderm. Embryos were injected with the dominant-negative BMP receptor (tBR) (2 ng) with or without *Xmad1* (M1) mRNA (2 ng). At stage 39, animal caps were photographed (left column) or sectioned for histology (right column). The truncated BMP receptor does not block formation of ventral mesoderm by Xmad1, as demonstrated by the presence of vesicles (V), mesenchyme, and mesothelium (Me).

(B) The dominant-negative BMP receptor blocks the Xmad1 induction of globin. Embryos were injected with tBR (2 ng), *Xmad1* (2 ng), or *Xmad1* (M1) mixed with tBR (2 ng of each), and at stage 39 animal cap RNA was analyzed as described in the legend to Figure 3.

(C) Xmad1 reverses the effects of the truncated receptors. Embryos were injected with the dominant-negative BMP receptor (tBR) (4 ng) with or without *Xmad1* (M1) mRNA (2 ng) or with the dominant-negative activin receptor (tAR) (2 ng) with or without *Xmad1* (M1) mRNA (2 ng). The truncated receptors, by blocking TGFβ signals, lead to expression of N-CAM. Coexpression of Xmad1 reverses this effect.

(D) A dominant-negative activin receptor (tAR) does not block Xmad2 induction of dorsal mesoderm. Embryos were injected with a dominant-negative activin receptor (tAR) (2 ng), *Xmad2* (2 ng), or *Xmad2* (M2) mixed with tAR (2 ng of each), and animal caps were cultured until either gastrula (Early) or tadpole (Late) stages. tAR is active as demonstrated by the expression of N-CAM and when coexpressed does not block formation of mesoderm (brachyury and muscle actin) by Xmad2.

an integrating point in the signaling pathway. It remains to be determined precisely how the other subsets of the TGFβ superfamily will be divided among the various Xmads. There are at least two other maternal Xmads (Xmad3 and Xmad4) in Xenopus, and these have yet to be functionally associated with TGFβ signals. Relatedly, it remains to be determined whether there are more *Xmad*-related genes in flies and worms and how these might correspond to TGFβ superfamily ligands in those organisms. The number of mammalian Xmad proteins is yet to be determined, but their clinical significance is highlighted by a recent report on human pancreatic cancers. Hahn et al. (1996) have identified a candidate tumor suppressor gene, *DPC4*, that is deleted in pancreatic cancers and have shown its sequence homology to *Mad* and *CEM* genes. Sequence comparison suggests that DPC4 is the human homolog of the molecule that we term Xmad4.

With respect to understanding mesoderm induction in Xenopus, our results show no differences in the distribution of maternal or zygotic *Xmad* mRNAs, and presumably their corresponding proteins are uniformly distributed along the future body axes. In other words, all cells in the marginal zone of early embryos are in principle capable of responding to either a dorsal mesoderm– or ventral mesoderm–inducing signal by virtue of having *Xmad1* and *Xmad2* mRNAs. Thus, a BMP signal is likely to activate Xmad1 on the ventral side of the embryo, whereas a dorsal-inducing signal (possibly Vg1 or activin) activates Xmad2 on the future dorsal side.

An unexpected finding is that formation of ventral mesoderm by Xmad1 occurs in the absence of brachyury expression (Figure 3). Xmad1 may directly activate differentiation for ventral mesoderm and not require expression of brachyury. Indeed, while brachyury is considered to be a general marker for embryonic mesoderm, there is no experiment that demonstrates that all mesoderm formation requires brachyury expression. In what may be a parallel example, the gene *neuroD* can apparently bypass early inhibitory influences that prevent neurogenesis in Xenopus and directly convert animal cap cells to neurons (Lee et al., 1995).

All the injections reported in this paper were done with mRNAs encoding wild-type Xmad proteins, not mutant or constitutively active forms. Why does injection of wild-type *Xmad* mRNA, which is already present in the embryo, lead to formation of mesoderm? Evidently, injection of *Xmad* mRNA leads to production of active Xmad protein, and this could occur by a number of mechanisms. Animal cap cells have endogenous BMP and activin mRNAs and are presumably exposed to a low level of the BMP and activin signaling pathways,

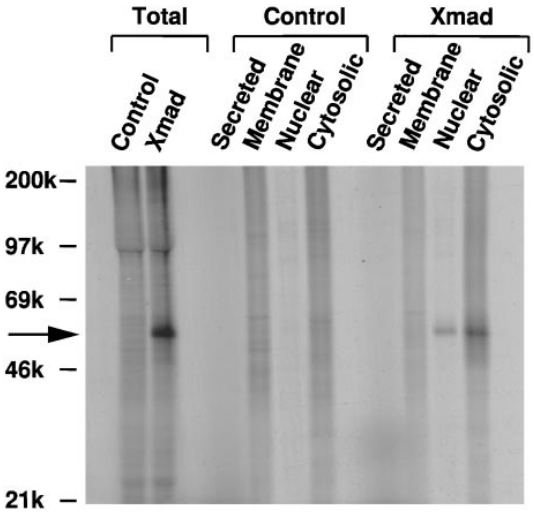

Figure 7. Xmad Proteins Are Present in the Nucleus and Cytosol

Stage-6 oocytes, injected with 30 ng of *Xmad* mRNA and cultured in media containing ^{35}S-labeled amino acids, were fractionated, and total, secreted, membrane-associated, nuclear, or cytosolic proteins were analyzed by SDS–polyacrylamide gel electrophoresis. The total homogenate demonstrates that *Xmad*-injected oocytes contain a specific Xmad protein band (arrow). Fractionation demonstrates that the Xmads are located in the cytosol and the nucleus. This figure shows the results obtained with *Xmad2*; identical results were obtained with *Xmad1*.

albeit at levels insufficient to induce mesoderm (Hemmati-Brivanlou and Melton, 1992; Graff et al., 1994; Hawley et al., 1995; Sasai et al., 1995; Schmidt et al., 1995; Wilson and Hemmati-Brivanlou, 1995). The ectopic expression of Xmad, combined with these constitutive pathways, may increase the level of signaling (BMPs for Xmad1 and activin/Vg1/nodal for Xmad2), leading to induction of mesoderm. Another possibility is that the Xmads are under negative regulation and supplying excess Xmad protein may overwhelm this control. Similar to our results with the Xmads, mRNA injection of some components of the Wnt signal transduction pathway, such as glycogen synthase kinase-3 or dishevelled, leads to activation of the Wnt signal (He et al., 1995; Pierce and Kimelman, 1995; Sokol et al., 1995).

As mentioned above, Xmads appear to be points at which information is integrated, in that each Xmad conveys the input from a subset of TGFβ superfamily ligands. There is another sense in which the Xmads may be involved in integrating information, namely in measuring the amount of signal that a cell receives. When Xenopus blastula cells are exposed to different concentrations of activin, different kinds of dorsal mesoderm are produced (Green et al., 1990, 1992; Wilson and Melton, 1994). For example, high concentrations produce notochord and lower concentrations produce muscle. Similarly, different amounts of Xmad2, presumably reflecting different amounts of Xmad2 activity, lead to expression of markers of different types of mesoderm (Figure 4). Therefore, it is possible that Xmads are the counting device used by cells to measure the concentration of ligand. For example, a posttranslational modification such as phosphorylation could control the nuclear:cytoplasmic ratio of Xmads. Alternatively, the activity of an individual Xmad may be determined by the number of phosphorylated residues, which in turn reflects the concentration of the ligand. Determining whether any of these biochemical mechanisms regulate Xmad activity may help us understand how morphogenetic signals control cell fates during development.

Experimental Procedures

Cloning *Xmad* cDNAs

To identify vertebrate genes related to the Drosophila gene, *Mad*, and the C. elegans sequences *CEM-1*, *CEM-2*, and *CEM-3*, two degenerate oligonucleotides were used as PCR primers. The primers are flanked by an BamHI or EcoRI linker, 5′ and 3′ respectively.

The sequence of the primers, 5′ to 3′, are: CGGGATCCTIGA(CT)GGI(AC)GI(TC)TICA(AG)(AG)T and CGGAATTCTA(AG)TG(AG)TAIGG(AG)TT(TGA)AT(AG)CA . These primers were used to amplify a fragment from Xenopus embryonic cDNA. RNA was isolated from embryos, and the first strand of the cDNA was synthesized with MMLV reverse transcriptase and oligo(dT). The cDNA was used as a template for the PCR with the following conditions: 1 cycle of 93°C for 3 min, 42°C for 1.5 min, 72°C for 1 min; then 4 cycles of 93°C for 1 min, 42°C for 1.5 min, 72°C for 1 min; followed by 30 cycles of 93°C for 1 min, 55°C for 1.5 min, 72°C for 1 min; and finally 1 cycle of 72°C for 5 min. The PCR fragments obtained were subcloned into pBluescript KS(II) (Stratagene). The PCR fragments were sequenced and individual clones were used as a probe to screen a Xenopus oocyte cDNA library (Rebagliati et al., 1985). The entire proceedure was performed as described previously (Graff et al., 1994). The cDNAs from the library were sequenced on both strands. A Xmad2 clone was obtained that lacked the exon in brackets in Figure 1.

Formation of Synthetic mRNA for Microinjection

To make synthetic mRNA encoding Xmad proteins, pSP64T-derived plasmids containing the entire *Xmad* cDNA were linearized with XbaI and transcribed in vitro as described previously (Krieg and Melton, 1987). The clones are termed pSP64TNE-Xmad1 (also known as pSP64TNE-545-1) and pSP64TNE-Xmad2 (also known as pSP64TNE-545-4). Synthetic mRNA encoding a truncated type I BMP receptor (tBR) (Graff et al., 1994) and a truncated type II activin receptor (tAR) (Hemmati-Brivanlou and Melton, 1992) are described elsewhere. Embryos were either uninjected (control) or injected with the indicated amounts of mRNA.

Embryological Methods

Embryos were obtained, microinjected, and cultured and animal caps were dissected as described previously (Thomsen and Melton, 1993; Graff et al., 1994). Histological sections were cut from parrafin-embedded samples and stained with geimsa for photography (as in Graff et al., 1994). All embryonic stages are according to Nieuwkoop and Faber (1967). Mesoderm-inducing proteins were added to a buffer consisting of 0.5× MMR and 0.5% bovine serum albumin. Activin was a gift of Dr. Mather at Genentech. BMP4 was provided by Dr. Celeste of Genetics Institute.

Analysis of RNA by RT–PCR

Proteinase K digestion, RNA extraction, and RT–PCR analyses have been described previously (Graff et al., 1994; Wilson and Melton, 1994). The intensities of the radioactive bands amplified by RT–PCR reflects the abundance of the mRNA (Graff et al., 1994; Wilson and Melton, 1994), and this was verified for these experiments by varying the amounts of cDNA template and confirming that the intensity of the band corresponds to the abundance of the mRNA (data not shown). In each experiment (Figures 3–6), the PCR-amplified products in each lane represent a fraction (approximately 2%) of the RNA isolated from a pool of animal caps.

The conditions of the PCR detection of RNA transcripts and the sequences of most of the primers have been previously described for brachyury, goosecoid, muscle actin, N-CAM, EF-1α, and globin (Graff et al., 1994; Hemmati-Brivanlou and Melton, 1992; Wilson and Melton, 1994). The primer sequences that have not been previously

described are listed below 5′ to 3′, and both primer sets were used for 25 cycles.

Xmad1 upstream, ACA GCA GCA TTT TTG TTC AG; *Xmad1* downstream, GAG ACC GAG GAG ATG GGA TT; *Xmad2* upstream, TCC CCT TCA GTC CGC TGC; *Xmad2* downstream, CCA ACA AGG TGC TTT TCG.

Oocyte Injection and Protein Fractionation

Stage VI ocytes were isolated, injected with 30 ng of *Xmad* mRNA, and cultured in media containing ^{35}S-labeled amino acids to label newly translated proteins as described previously (Smith et al., 1991b; Kessler and Melton, 1995). The culture medium containing the secreted proteins was isolated (Smith et al., 1991b; Kessler and Melton, 1995). Oocytes were homogenized at 4°C in buffer 94A+ (0.25 M sucrose, 20 mM HEPES [pH 7.4], 50 mM KCl, 0.5 mM $MgCl_2$, 1 mM K–EGTA [pH 7.4], 1 mM PMSF, 1 μg/ml leupeptin), and this fraction is termed total in Figure 6. After removing the yolk by low speed centrifugation at 1000 × g for 5 min at 4°C, the membrane and cytosolic fractions were isolated by centrifugation at 100,000 × g for 45 min at 4°C (Evans and Kay, 1991). The nuclei were isolated by manual dissection (Evans and Kay, 1991). One oocyte equivalent of each compartment was analyzed by 10% SDS–PAGE in the presence of the reducing agent dithiothreitol.

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